Background—The mechanisms responsible for tumor necrosis factor (TNF)–induced LV structural remodeling in the adult heart are not known.

Methods and Results—We generated a line of transgenic mice (MHCsTNF) with cardiac restricted overexpression of TNF that develop progressive LV dilation/remodeling from 4 to 12 weeks of age. During the early phases of LV structural remodeling, there was a significant increase in total matrix metalloproteinase (MMP) activity that corresponded to a decrease in total myocardial fibrillar collagen content. As the MHCsTNF mice aged, there was a significant decrease in total MMP zymographic activity that was accompanied by an increase in total fibrillar collagen content. The changes in total MMP activity and myocardial fibrillar collagen content were related to a time-dependent increase in myocardial tissue inhibitor of metalloproteinases (TIMP)-1 levels, resulting in a significant time-dependent decrease in the MMP activity/TIMP level ratio in the MHCsTNF mice. To determine a possible mechanism for the increase in myocardial fibrosis, we also measured levels of TGF-β1 and TGF-β2 protein levels, which were shown to be significantly elevated in the hearts of the MHCsTNF mice.

Conclusions—Our results suggest that progressive time-dependent changes in the balance between MMP activity and TIMP activity are responsible, at least in part, for the spectrum of TNF-induced changes in the myofibrillar collagen content that occur during LV structural remodeling in the MHCsTNF mice. (Circulation. 2001;104:826-831.)

Key Words: heart failure n genes n growth substances n collagen n tumor necrosis factor

Although the classic paradigms that have been used to explain the development and progression of left ventricular (LV) remodeling have focused on the mechanisms that are responsible for provoking increased fibrillar collagen content in the myocardium, it has always been difficult to reconcile how increased myocardial fibrosis, which would be expected to lead to a stiffer and less compliant ventricle, contributed to the progressive LV dilation that occurs during the process of LV remodeling. It has recently become apparent that a family of collagenolytic enzymes, called matrix metalloproteinases (MMPs), are expressed in the myocardium during LV structural remodeling. Conceptually, progressive activation of MMPs would be expected to facilitate progressive degradation of fibrillar collagen, which would in turn facilitate mural realignment of myofibrillar bundles, thus leading to increased LV wall thinning and LV dilation. And indeed, experimental studies have shown that pharmacological inhibition of MMPs attenuates LV dilation in experimental heart failure models. Nonetheless, although the concept of MMP-induced degradation of fibrillar collagen represents a plausible mechanism for the LV dilation that occurs during the remodeling process, activation of MMPs does not readily explain the development of myocardial fibrosis, which is the hallmark of the end-stage remodeling process. Thus, the mechanisms that provoke both increased LV dilation and increased myocardial fibrosis are not at all clear.

Relevant to the above discussion is the observation that molecules that are expressed within the heart after cardiac injury, such as tumor necrosis factor (TNF), are capable of upregulating MMP activity. In previous studies, TNF up-regulation has been shown after infarction in areas of the myocardium where LV dilation and wall thinning are occurring. Furthermore, short-term administration of TNF resulted in increased LV dilatation and LV wall thinning in experimental animals. Moreover, the TNF-induced changes in LV...
structural remodeling were accompanied by a loss of myocardial fibrillar collagen in the myocardium, consistent with a TNF-induced increase in MMP expression. In contrast, longer-term studies in transgenic mice with targeted cardiac overexpression of TNF have consistently shown that the older mice develop progressive myocardial fibrosis. Taken together, these observations suggested the interesting possibility that TNF might provoke a spectrum of changes that are important in LV structural remodeling. To address this question, we have developed a line of transgenic mice with cardiac restricted overexpression of TNF to delineate the effects of sustained TNF expression on the factors that lead to degradation of fibrillar collagen, namely MMP activation, as well as the factors that favor fibrosis, namely tissue inhibitors of matrix metalloproteinases (TIMPs).

Methods

Generation and Characterization of Transgenic Mice With Cardiac Restricted Overexpression of TNF

Generation of MHCsTNF Transgenic Mice

The methods used to generate transgenic mice with targeted cardiac overexpression of TNF (referred to as MHCsTNF mice) were reported previously. The MHCsTNF line, which was originally developed in a C57BL6 strain of mice, was subsequently bred with pure ICR and hybrid C57/BL6 × ICR strains to expand the colonies. Age-matched littermate controls lacking the transgene were used as the appropriate controls.

Characterization of the MHCsTNF Mice

Transgene expression was assessed by Northern blot analysis of total RNA from various tissues, including brain, spleen, liver, lung, and heart, as described. The level of TNF protein in myocardial extracts and in the peripheral circulation of the MHCsTNF mice and littermate controls was determined by ELISA (Quantikine, R&D Systems). Similarly, levels of TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) protein were quantified in myocardial extracts from the MHCsTNF mice and littermate controls by ELISA (FactorTest-X, Genzyme Diagnostics). In preliminary control experiments, we showed that the ELISA for TNFR1 and TNFR2 did not detect TNFR1 and TNFR2 protein levels, respectively, in TNFR1- and TNFR2-knockout mice (data not shown). The methods used to determine LV end-diastolic dimension by 2D directed M-mode echocardiography have been described.

Myocardial Ultrastructure in MHCsTNF Mice

Transmission and scanning electron microscopy and picrosirius red staining were performed on hearts from MHCsTNF mice and littermate controls that were harvested at 4, 8, and 12 weeks of age (see Data Supplement online at http://www.circulationaha.org).

MMP and TIMP Activity in MHCsTNF Mice

MMP Activity

MMP activity was measured at 4, 8, and 12 weeks of age in myocardial extracts from MHCsTNF mice and littermate controls by gelatin zymography. Zymograms were digitized with a Kodak DCS 420 digital camera (Kodak Inc.), which provides high-resolution (1500×1000 pixels) and consistent exposure control between scans. The proteolytic regions for each sample were determined by quantitative image analysis, and the final results were expressed as pixel density per lane. Zymographic analysis was performed in duplicate for all samples by use of identical protein concentrations.

Expression of TIMPs

TIMP-1 levels were measured at 4, 8, and 12 weeks of age in myocardial extracts from MHCsTNF mice and littermate controls. TIMP-1 levels were measured by ELISA (Amersham RPN 2611), according to the manufacturer’s suggestions.

Expression of Fibrogenic Cytokines

The expression of transforming growth factor (TGF)-β1 and TGF-β2 was examined in the MHCsTNF and littermate control mice at 4, 8, and 12 weeks of age. For these studies, mRNA levels for TGF-β1 and TGF-β2 were measured with ribonuclease protection assays (Pharmingen). TGF-β1 and TGF-β2 protein levels were measured in myocardial extracts (see above) by ELISA (Quantikine), according to the manufacturer’s suggestions.

Statistical Analysis

Each value is expressed as mean ± SEM. Two-way ANOVA was used to test for mean differences in LV end-diastolic dimension, TNF and TNF receptor protein levels, fibrillar collagen content, MMP activity, TIMP-1 levels, the ratio of MMP activity/TIMP-1, and TGF-β1 and TGF-β2 levels in the MHCsTNF mice and littermate controls at 4, 8, and 12 weeks of age. Where appropriate, post hoc ANOVA testing (Tukey’s test) was used to assess mean differences between groups at a given time point. Data were subjected to logarithmic transformation before the parametric analysis, if they were not distributed normally. Significant differences were said to exist at a value of P < 0.05.

Results

Characterization of the MHCsTNF Mice

Figure 1A shows the Northern blot analysis of total RNA from various tissues in the MHCsTNF mice and littermate controls. The MHCsTNF transgene was expressed in the heart but was not detected in the liver, lung, spleen, or brain. Myocardial and circulating peripheral levels of TNF protein and TNF receptor proteins were measured in the MHCsTNF mice and littermate controls at 4, 8, and 12 weeks of age (see Data Supplement). TNF, TNFR1, and TNFR2 protein levels were significantly greater (P < 0.004) in the myocardial extracts and the peripheral circulation from the MHCsTNF mice than in littermate controls. There was no significant (P > 0.05) difference, however, between male and female transgenic mice or between male and female littermate control mice (see Data Supplement) with respect to the myocardial levels of TNFR1 and TNFR2.

Figure 1B shows the gross pathology and histology of the MHCsTNF mice and littermate controls at 4 and 8 weeks of age. As shown, the MHCsTNF mice developed progressive cardiomegaly at 8 and 12 weeks of age compared with the littermate controls lacking the transgene. Cross-sectional examination of the heart revealed both right and left ventricular dilation, with LV wall thinning in the MHCsTNF mouse. Histological examination of the hearts from the MHCsTNF mice revealed significant myofibrillar disarray throughout the myocardium. There were also scattered areas of infiltrating interstitial cells in the MHCsTNF mice; however, this was not a prominent aspect of the histological appearance of the mice. Finally, there was a significant (P < 0.02) overall increase in LV dimensions from 4 to 12 weeks of age in the MHCsTNF mice compared with littermate controls.

Myocardial Ultrastructure in the MHCsTNF Mouse

Representative transmission electron micrographs of the LV myocardial samples from littermate controls and MHCsTNF mice are depicted in Figure 2, A through C. The transmission electron micrographs from the littermate control mice at 4
weeks (Figure 2A) revealed a characteristic linear array of sarcomeres and myofibrils. In contrast, the myofibrils in the 4-week-old MHCsTNF mice were less organized, with loss of sarcomeric registration observed in many of the sections (Figure 2B). The ultrastructural abnormalities in the MHCsTNF mice were further exaggerated in the 12-week-old MHCsTNF mice, which showed a significant loss of sarcomere registration and myofibril disarray (Figure 2C). To delineate the changes in the extracellular matrix that accompanied the loss of sarcomere registration and myofibril disarray (Figure 2C), we next performed scanning electron microscopy (Figure 2, D through F). The salient finding shown by Figure 2E is that there was a significant loss of fibrillar collagen in the MHCsTNF mice at 4 weeks of age compared with age-matched littermate controls (Figure 2D). As the MHCsTNF aged (12 weeks), however, there was an obvious increase in myocardial fibrillar collagen content. In other areas of the myocardium (data not shown), the fibrillar collagen weave in the hearts of the MHCsTNF mice remained disrupted and poorly organized compared with littermate controls. Thus, the LV structural remodeling observed in the MHCsTNF mice was accompanied by alterations in myocardial collagen structure and organization, as well as progressive misalignment of sarcomeres and myofibrils. Similar findings were observed in 3 additional pairs of age-matched MHCsTNF and littermate control hearts.

To further characterize the time-dependent changes in fibrillar collagen content, we performed picrosirius red staining in the MHCsTNF mice at 4, 8, and 12 weeks of age (n=5 hearts per time point). Figure 3 shows that LV myocardial fibrillar collagen content was significantly (P<0.01) reduced as well as progressive misalignment of sarcomeres and myofibrils. Similar findings were observed in 3 additional pairs of age-matched MHCsTNF and littermate control hearts.

Figure 1. Characterization of MHCsTNF mice. A, Northern blot analysis of total RNA from various tissues in MHCsTNF mice and littermate controls. B, Gross pathology and histology of MHCsTNF mice and littermate controls at 4 and 8 weeks of age. C, Results of 2D directed M-mode echocardiographic examination of LV dimensions in MHCsTNF mice and littermate controls at 4, 8, and 12 weeks of age. Two-way ANOVA showed significant overall differences (P<0.02) in LV end-diastolic dimension between groups; post hoc testing showed significant (P<0.003) differences between MHCsTNF and littermate control mice at 8 and 12 weeks of age. LVEDD indicates LV end-diastolic dimension; *P<0.05 vs littermate control mice.

Figure 2. Transmission and scanning electron micrographs. A through C, Representative transmission electron micrographs in littermate controls (A) and MHCsTNF mice at 4 (B) and 8 (C) weeks of age. Two-way ANOVA showed significant overall differences (P<0.02) in LV end-diastolic dimension between groups; post hoc testing showed significant (P<0.003) differences between MHCsTNF and littermate control mice at 8 and 12 weeks of age. LVEDD indicates LV end-diastolic dimension; *P<0.05 vs littermate control mice.

Figure 3. Myocardial fibrillar collagen content. Total myocardial fibrillar collagen content was determined by picrosirius red technique in MHCsTNF and littermate control mice at 4, 8, and 12 weeks of age (see text for details). Two-way ANOVA revealed a significant difference between groups (P<0.01); post hoc ANOVA testing showed that there was a significant (P<0.01) decrease in fibrillar collagen in MHCsTNF mice at 4 weeks of age and a significant (P<0.05) increase in fibrillar collagen at 12 weeks of age.
Figure 4. MMP activity and TIMP levels. A, Representative gelatin zymogram depicting total MMP activity in MHCsTNF and littermate control mice at 4, 8, and 12 weeks of age; B, summarized results of group data for total MMP zymographic activity. Two-way ANOVA showed significant (P<0.001) overall differences in total MMP zymographic activity between MHCsTNF and littermate control mice; post hoc ANOVA showed that MMP activity was significantly (P<0.001) greater in MHCsTNF mice at 4 weeks of age. C, Time-dependent changes in TIMP levels at 4, 8, and 12 weeks in MHCsTNF and littermate control mice. Two-way ANOVA showed significant interaction (P<0.034) between MHCsTNF mice and littermate controls with respect to TIMP-1 levels; post hoc ANOVA showed that TIMP-1 levels were significantly (P<0.05) less in MHCsTNF mice at 4 weeks of age. D, Time-dependent changes in ratio of MMP activity/TIMP levels in MHCsTNF and littermate control mice. Two-way ANOVA showed significant differences in this ratio between groups (P<0.001); post hoc ANOVA showed significant (P<0.001) differences at 4 weeks of age, whereas this ratio was not significantly different at 8 and 12 weeks. LM indicates littermate control; TG, MHCsTNF transgenic; *P<0.05 vs littermate control mice.

at 4 weeks in the MHCsTNF mice compared with littermate controls, coinciding with the qualitative changes in fibrillar collagen weave observed at 4 weeks in the MHCsTNF mice (Figure 2D). As the MHCsTNF mice aged, however, there was a time-dependent increase in myocardial fibrillar collagen content, with the result that the fibrillar collagen content was significantly (P<0.05) greater in the MHCsTNF mice by 12 weeks of age. Thus, LV structural remodeling in the MHCsTNF mice was accompanied by biphasic changes in fibrillar collagen content.

MMP and TIMP Activity in MHCsTNF Mice

Activation of MMPs

Figure 4A shows a representative gelatin zymogram depicting MMP activity in the littermate controls and MHCsTNF mice at 4, 8, and 12 weeks of age. As shown, the lytic bands corresponding to total MMP activity were more prominent in the MHCsTNF mice at 4 weeks of age. The lytic bands at ~65 kDa are suggestive of MMP-2 and/or MMP-9 activity. Figure 4B, which summarizes the results of group data (n=6 hearts per group per time period), shows 2 important findings with respect to total MMP zymographic activity in the MHCsTNF mice. First, MMP zymographic activity was significantly (P<0.001) greater in the MHCsTNF mice at 4 weeks of age, coinciding with the decrease in fibrillar collagen content observed in the mice at this time point.

Second, there was a striking decrease in MMP zymographic activity in the MHCsTNF mice from 8 to 12 weeks of age, which corresponded to the increase in fibrillar collagen content observed in the MHCsTNF mice at these time points. There was no significant (P>0.05) difference in MMP activity between the MHCsTNF and littermate control mice at 8 and 12 weeks of age.

TIMP Levels

The salient finding shown by Figure 4C is that TIMP-1 levels increased progressively from 4 to 12 weeks of age in the MHCsTNF mice (n=6 hearts per group per time period), whereas TIMP-1 levels remained relatively constant in the littermate control mice until 12 weeks of age (P=0.034). To determine the relationship between MMP activity and TIMP levels, we next examined the ratio of total MMP zymographic activity/TIMP-1 levels. As depicted in Figure 4D, the ratio of total MMP zymographic activity/TIMP-1 levels was significantly (P<0.001) elevated in the MHCsTNF mice at 4 weeks of age, consistent with the decrease in collagen content observed at this time. Moreover, there was a striking decrease in this ratio at 8 and 12 weeks of age in the MHCsTNF mice, consistent with the progressive increase in myocardial fibrosis observed in these mice during this time period.

Expression of Fibrogenic Cytokines

Figure 5A shows the results of ribonuclease protection assays for TGF-β1 and TGF-β2 mRNA levels in MHCsTNF and littermate controls (n=2 hearts per group per time period). The relative levels of TGF-β1 and TGF-β2 mRNA were ~4-fold and 6-fold greater, respectively, in the MHCsTNF at all time points tested. Figure 5B and 5C show, respectively, the myocardial levels of TGF-β1 and TGF-β2 protein (n=5 hearts per group per time period) in the MHCsTNF mice and
Given that TNF is known to upregulate the expression of different fibrogenic cytokines/proteins, the present study is the first to demonstrate a relationship between selective myocardial TNF expression and MMP/TIMP stoichiometry.

**Discussion**

The results of the present study suggest that time-dependent changes in the balance between MMP activity and TIMP levels are responsible, at least in part, for the spectrum of changes that occur in the extracellular matrix during LV structural remodeling in MHCsTNF mice. That is, during the early phases of LV structural remodeling in the MHCsTNF mice (Figure 1B and 1C), there was an increase in total MMP activity (Figure 4B); this increase, however, was not accompanied by a concomitant rise in TIMP-1 levels (Figure 4C). Interestingly, these changes in the extracellular matrix occurred before the onset of LV dilation in the MHCsTNF mice (Figure 1C). As the MHCsTNF mice aged from 8 to 12 weeks, there was a significant decrease in total MMP activity that was accompanied by an increase in TIMP-1 levels and a decrease in the ratio of MMP activity/TIMP levels (Figure 4). These changes in MMP/TIMP stoichiometry were associated with an increase in total fibrillar collagen content (Figures 2F and 3). Importantly, there was no further increase in LV dilation in the MHCsTNF mice after the increase in myocardial fibrillar collagen content at 4 weeks of age in the MHCsTNF mice (Figures 2E and 3). Importantly, these changes in the extracellular matrix occurred before the onset of LV dilation in the MHCsTNF mice (Figure 1C). As the MHCsTNF mice aged from 8 to 12 weeks, there was a significant decrease in total MMP activity that was accompanied by an increase in TIMP-1 levels and a decrease in the ratio of MMP activity/TIMP levels (Figure 4). These changes in MMP/TIMP stoichiometry were associated with an increase in total fibrillar collagen content (Figures 2F and 3). Importantly, there was no further increase in LV dilation in the MHCsTNF mice after the increase in myocardial fibrillar collagen content occurred, although the overall LV dimensions remained significantly greater in the MHCsTNF mice than in littermate controls (Figure 1C). Thus, the results of the present study provide a mechanistic basis for reconciling the differences between studies that have shown that short-term effects of TNF on TIMP expression are exceedingly complex, and the context within which TNF signaling occurs. An alternative and perhaps more likely explanation for the temporal changes in TIMP expression is that the effects of TNF were mediated indirectly through a time-dependent expression of ≥1 different fibrogenic cytokines/proteins.

**Role of MMP and TIMP Expression in LV Structural Remodeling**

The present study is the first to demonstrate a relationship between selective myocardial TNF expression and MMP/TIMP profiles and the process of LV structural remodeling. Given that TNF is known to upregulate the expression of MMPs and that TNF increases the expression of neutral serine proteases that activate MMPs, it was perhaps not surprising that we observed increased MMP activity in the MHCsTNF mice. What was not predicted, however, was the progressive decrease in MMP activity and the progressive rise in TIMP levels as the MHCsTNF mice aged. Although the mechanism(s) for the increased TIMP expression is not known, at least 2 potential explanations warrant discussion. First, it is possible that sustained overexpression of TNF led to a direct increase in TIMP levels in the MHCsTNF. The effects of TNF on TIMP expression are exceedingly complex, however, and are influenced by cell type, TNF concentration, and the context within which TNF signaling occurs. An alternative and perhaps more likely explanation for the temporal changes in TIMP expression is that the effects of TNF were mediated indirectly through a time-dependent expression of ≥1 different fibrogenic cytokines/proteins. Germaine to this discussion was the finding that TGF-β1 and TGF-β2 protein levels were significantly increased in the hearts of the MHCsTNF mice (Figure 5). Given that TNF is known to upregulate the expression and release of TGF-β2 and that TGF-β increases both TIMP-1 expression and increased collagen synthesis, these findings may provide one potential explanation for the increase in myocardial collagen content in the MHCsTNF mice from 8 to 12 weeks of age. A second possible explanation is that the TNF-induced changes in LV dimension and wall thickness led to an increase in LV wall stress and that changes in LV wall stress were responsible for the increased expression of fibrogenic molecules. The results of the present study differ somewhat from a previous report by Li et al., who observed persistent MMP expression, myocardial fibrosis, and cardiac dilatation in a model of targeted TNF overexpression. Although the reasons for the minor discrepancies between these 2 studies are not clear, they may relate to different genetic backgrounds, different levels of TNF protein expression, or the different degrees of inflammatory infiltrates in the 2 models.

**Conclusions**

The findings of the present experimental study underscore the extremely complex and dynamic nature of the biological changes that occur within the extracellular matrix after sustained and/or chronic myocardial injury/inflammation. It bears emphasis that our findings in the remodeled hearts of the MHCsTNF mice are entirely consistent with experimental models of chronic injury/inflammation in an array of different organs, including liver, lung, and kidney, in which an initial increase in MMP expression is superseded by the increase in fibrogenic cytokines (eg, TGF-β), increased TIMP expression, and progressive tissue fibrosis. Implicit in these statement is the suggestion that attempts to prevent and/or modulate LV structural remodeling through alterations in MMP activity will need to consider the dynamic and time-dependent nature of the changes that occur in MMP activity, TIMP activity, and fibrogenic cytokine expression during the remodeling process. For example, strategies designed to inhibit MMP activity during the early stages of the remodeling process when the MMP/TIMP ratio is likely to be highest may be beneficial by preventing LV dilation from occurring. And indeed, this has been shown to be the case experimentally. Strategies designed to inhibit MMP activity during the latter stages of the remodeling process, in which the MMP/TIMP ratio is relatively lower, however, may prevent further LV dilation, albeit at the expense of accelerated myocardial fibrosis and increased myocardial stiffness. Accordingly, in future studies it will be important to understand the basic mechanisms that govern the interplay between MMP and TIMP expression after sustained and/or chronic myocardial injury/inflammation.
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